STUDY OF CRYPHONECTRIA PARASITICA, CHESTNUT BLIGHT, IN EAST TEXAS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Study of *Cryphonectria parasitica*, Chestnut Blight, in East Texas

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The iconic fungal pathogen *Cryphonectria parasitica*, the causal agent of chestnut blight, is known for the phenomenon of hypovirulence attributed to dsRNA. Hypovirulence is a trait caused by infection of the fungus with a virus that debilitates the pathogen and is proposed as a potential biocontrol for chestnut blight. *C. parasitica* has not been officially studied in Texas even though the pathogen has been established a lives on native tree species in eastern Texas. Studies were done by characterizing the morphology and virulence of isolates collected from a tree in Tatum, Texas and compared to known a known hypovirulent isolate and a wild type. The East Texas isolates contained abnormal morphology that are associated traits of hypovirulence such as sectoring, reduced growth, and discoloration. The East Texas isolate (designated Tatum) grew significantly more slowly *in vitro* than the wild type isolate but more quickly than the known hypovirulent isolate. The virulence assay on apples also found the Texas isolate to be less virulent than the wild type but more virulent than the hypovirulent isolate. These studies could not disprove that the Tatum isolate from East Texas is infected with the hypovirulent virus and will provide a basis for further investigations on the status of the Texas *C. parasitica* population.

DEDICATION

I dedicate this thesis to my mother for always pushing me to pursue my dreams, and to my fiancé for encouraging me quit my second job so that I could put all my energy into school. I would also like to dedicate this to my teachers and professors who have helped develop me into the person that I have become. Lastly, I dedicate this to all the American chestnut trees surviving by their roots in the eastern forests of America

ACKNOWLEDGMENTS

I would like to acknowledge Les Jameson & family for making contact with us and inviting us to take samples of his tree in Tatum, Texas and sharing their stories and kindness. I would like to acknowledge Shelia McBride for helping sample the tree in Tatum. I would lastly like to acknowledge Albre Brown for patiently dedicating her time and guidance. I want to also acknowledge her for the assistance she provided in helping me learn a range of lab techniques, as well as allowing me to accompany her to her graduate level classes when I was able to.

CHAPTER I

INTRODUCTION

Before the 1900's, the American chestnut (*Castanea dentata*) towered over the canopies of the eastern forest of the United States. In 1904, a fungal pathogen by the name of *Cryphonectria parasitica*, the cause of Chestnut Blight, came to America on the imported seeds of Asian Chestnut trees in the Bronx Zoological Park in New York City (Merkel 1905). Chestnut blight made its way across the natural habitat of the American chestnut rapidly in forty years**,** killing 4 billion American chestnut trees in the process. The American chestnut was once the most abundant and largest tree in the North American eastern deciduous forest. Chestnut blight not only changed the whole ecology of the forest by eliminating a keystone species, but it changed the culture of the people who lived near it as well. The local people relied on the American chestnut for timber, food, furniture making, and the tannin industry (Anagnostakis 1992).

C. parasitica is an ascomycete that starts primary infection by entering a wound and infecting the phloem and cambium of the tree. The pathogen pumps oxalic acid that lowers the pH of the eventually girdling the tree (Anderson 1914). This ends up killing everything above the canker. The cankers may not be obvious on mature bark. The first noticeable sign of infection is the formation of epicormic shoots below the canker from the tree trying to avoid the blockage the pathogen has caused (Anagnostakis 1987). The secondary infection begins when the conidia are disseminated by wind, rain, and wildlife. Wildlife can carry the conidia to remote distances making quarantine impossible (Rand 1920). Virulent strains of *C. parasitica* can have conidia

that have been known to overwinter in the cambium of dead trees for up to 50 years (Anagnostakis 1987). *C. parasitica* also continues to live today as a widespread non-lethal pathogen on many types of oak as well as different species of chestnut trees (Wallace 1970).

Despite the devastation, there is still hope in restoring the great eastern American forest to pre-blight conditions. The root systems of the massive American chestnut still send up shoots eager to thrive once again (Andrande 2005). Significant control efforts have been attempted by crossing the naturally resistant Asian chestnut varieties with the American chestnut. Up to the present day, these efforts have been wholly unsuccessful, because the gene that controls resistance seems to be linked to the significantly smaller size of the Asian chestnut varieties (Bernatzky 1992).

While the current outcome in America has been bleak, in Europe, on the contrary, *C. parasitica* has a natural enemy, a fungal virus named Cryphonectria hypovirus. Cryphonectria hypovirus is a double-stranded RNA (Day 1977). In 1950, scientist had discovered European chestnut, *Castanea sativa,* with what seemed to be healing cankers. They isolated the pathogen and found that it contained a less virulent form of *C. parasitica* that was then designated as the term "hypovirulent". The result of this work lead to a local naturalist in western Michigan to sending the bark of surviving but blighted American chestnut trees to the Connecticut Agricultural Experiment Station. The American strain was also identified as being hypovirulent (MacDonald 1991). These strains of the blight that have been infected with this hypovirus are associated with superficial cankers (Anagnostakis 1987). Many strains of hypovirus-infected *C. parasitica* have been shown to have vegetative compatibility. Complimentary compatibility types allow them to

transmit the dsRNA through cytoplasmic transmission. This trait makes these strains a promising method for biological control (Elliston 1984).

The cankers of trees caused by hypovirus-infected strains of *C. parasitica* do not reach the vascular tissue of the plant which ends up causing the devastating girdling (M. Gryzenhout 2006). This has been linked to lack or decrease of oxalate production in the hypovirulent strains (Havir 1983). In culture, hypovirus-infected *C. parasitica* has been noted to produce a range of different morphological characteristics such as sectoring, discoloration, and decreased growth rate (Chen 1995). Attempts have been made to introduce hypovirulent strains by artificial inoculation to the American chestnut. Sadly, these attempts were not successful in the long term (Anagnostakis 1986).

A different species of tree, the Allegheny chinquapin (*Castanea pumila*), present throughout East Texas, is closely related to the American chestnut. Chinquapin, like the American chestnut, is also susceptible to *C. parasitica (*Wallace 1970*)*. However, there are indications that many different species may carry varying levels of resistance. The Allegheny chinquapin is more resistant than the American chestnut to *C. parasitica*, but it has been found that very few chinquapin can survive for extended periods of time by consistently producing basal sprouts (Paillet 2012).

A man by the name of Les Jameson contacted Dr. David Appel (Professor, Dept. of Plant Pathology and Microbiology, TAMU, College Station, TX) in the fall of 2014 about a chinquapin tree that has been surviving in his yard since the 1940s despite being infected with *C.*

parasitica in Tatum, Texas. He stated that his field was once full of chinquapin trees and all quickly died when the blight arrived except for the one tree that was in the middle of the yard, heavily blighted, and had significant amounts of basal shoots sprouting around the base. None the less, the tree still produces a small amount of fruit each year. The fruit are burr-covered nuts similar to a chestnut but smaller. Mr. Jameson's family expressed how these chinquapin trees were a significant part of the childhood of many that grew up in the area before the blight. The family described a popular local game they called "Holly Golly," that children would play using the fruit of the chinquapin. Like the American chestnut, the chinquapin became a significant part of the culture as well as ecology of the area. The devastation of the Allegheny chinquapin has largely been cast in the shadow of the American chestnut.

The *C. parasitica* population in Texas hasn't been formally studied to any extent. This surviving tree is able to provide an insight to an unstudied population of *C. parasitica* in Texas that took a toll on the small town communities in eastern Texas and devastated a population of chinquapin trees. Understanding the Texas population of *C. parasitica* can also contribute additional knowledge to the large body of scientific information known about this pathogen.

The overall objectives of this project were to grow and compare samples from a Texas strain of *C. parasitica* to the known wild type and a known hypovirulent strain. By doing so we should gain insight of the basic characteristics into the pathogen to enhance a future sequencing study of the *C. parasitica* population in Texas for the possible identification of a new hypovirulent strain.

CHAPTER II

METHODS

Many strains of *C. Parasitica* have been identified and can be certified dsRNA free or certified to contain dsRNA. For the purposes of this experiment, 2 isolates were purchased from the American Type Culture Collection (ATCC, 10801 University Boulevard, Manassas, VA 20110). The first is the *C. parasitica* strain designated EP 155. EP 155 is certified to not contain any dsRNA, and was isolated by Dr. Sandra Anagnostokis (Connecticut Agricultural Exeperiment Station, New Haven, CT 69540) as an American virulent strain. The second is the *C. parasitica* strain designated EP 4. EP 4 is certified to contain a dsRNA. EP 4 was isolated by Dr. Jean Gerent in France. EP 4 is of Jeaune Regenere (JR) phenotype. JR is a very slow growing flat culture that only reproduces on a very aerial mycelium. JR cultures have a dsRNA that has been incorporated into the nuclear genome. This phenotype is not thought to have been a product of laboratory culture. It is also very stable for transferring (Anagnostakis 1984).

Sample collection

Samples, in the form of infected bark, were taken from the chinquapin tree, located in Tatum, TX and brought back to the lab. Pure cultures were obtained by first growing the pathogen on water agar then transferring a very small amount to potato dextrose agar (PDA), which was amended with lactic acid at the concentration of 1 mL/ L. Cultures were then transferred to PDA without the lactic acid amendment. The PDA cultures were grown in an incubator at 25 °C. Cultures were observed for growth patterns, growth rate (cm), and cultural morphology. The growth of ten transfers from each EP155, EP4, and the Tatum samples was recorded by tracking the mean

of the diameter taken both vertically and horizontally for each culture over time. The horizontal and vertical axes were determined by a pre-drawn vertical and horizontal axis on the bottom of the petri dish. The area that was labeled with the date and pathogen type was used as a reference for quadrant 4. Statistical analysis was performed using one-way ANOVA followed by a Tukey HSD test for day 5 on each trial to determine if there was a difference between the sizes of the cultures at a given time. The one-way ANOVA and Tukey HSD test were performed using an online calculator available from http://vassarstats.net/.

Apple virulence assay

Twelve granny smith apples of uniform size were selected and randomly placed into 1 of 4 treatment groups, control, EP155, EP4, or Tatum. All apples were wiped with a solution of 70% ethanol. A 7 mm cork borer was used to carefully break the skin and remove flesh about 3-5mm in depth. Then a 7 mm agar/mycelial plug that was removed from the culture of the pathogen was placed in the hole created by the cork borer. The specific mycelial/ agar plug placed in the apple depended on its treatment group. The control group received a plug that consisted of only PDA agar. The other groups received a plug that consisted of PDA agar and their respective assigned culture. The plugs were placed in the apples under a laminar flow hood. They were then sealed with parafilm and placed in a 30 cm by 15 cm plastic box. The plastic box was then placed in a dark incubator at 25°C for 3 days. On the 3rd day, the parafilm was removed and the boxes were placed on the lab bench top at room temperature. 5 days and 10 days after the agar/mycelial plugs were placed, the necrotic lesions were measured. Statistical analysis was performed using one-way ANOVA followed by a Tukey HSD test using an online calculator from http://vassarstats.net/. The apple virulence test was performed using methods described by

Elliston (Elliston 1984) with a few minor modifications that have been made by Polashock (Polashock 1994).

Oak branch inoculation

Forty dormant water oak branches approximately 30 cm long and 2 cm in diameter were cut down and the ends were sealed using unscented candle wax to preserve the life of the branch. Using a 7 mm cork borer, the bark was removed. They were then randomly selected to go into 1 of 4 treatment groups. Each treatment group was assigned 10 branches. Three of the treatment groups received a mycelium-agar plug with the corresponding culture grown on PDA of either EP 155, Tatum, or EP 4. The fourth group received an agar plug with only PDA to be used as a control group. Each excised branch was then wrapped in parafilm around the agar plug and placed in a plastic box and stored for 7 days on the bench top with damp paper towels to create a high humidity environment. After 7 days, the parafilm was removed and the branches were observed for 2 weeks for signs of sporulation, evidence of cankering, and damage from infection. After 20 days, the bark was removed from the branches around the inoculation sight to look for characteristic discoloration of the wood for signs of cankering. Any discoloration was measured and then compared to the other cultures for evidence of a virulence index on a native oak species. Methods used were described by Lee with modifications to how the edges of the branches were sealed (Lee 1992).

CHAPTER III

RESULTS

Culture observation

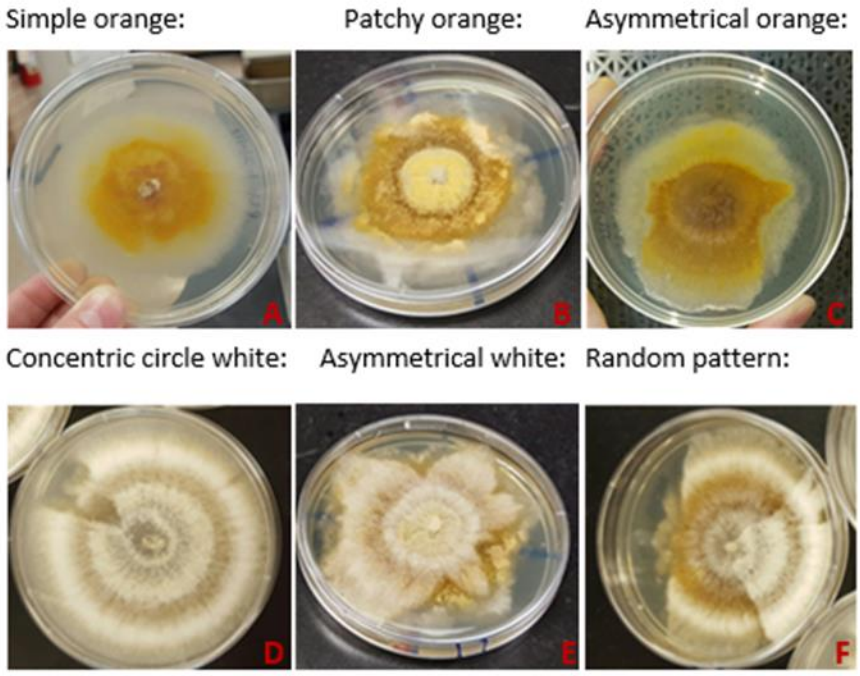

Figure 1. *C.parasitica* culture morphology variation. Examples and simple naming scheme of the culture morphology from the chinquapin tree in Tatum, Texas.

Culture morphology traits exhibited by the Tatum isolates can be seen in figure 1. Simple orange cultures (figure 1A) had mycelium that grew completely vegetative. The orange coloring stayed condensed in the middle as the hyphae grew out. Simple orange stayed relatively symmetrical throughout the maturing process. This culture stayed orange even after aging past 14 days and there was no discoloration of the media.

Patchy orange cultures (figure 1B) had a random pattern of patches of hyphae that grew thick and appeared fuzzy with a fuzzy circular center. This morphology also grew overall fairly symmetrical. After 14-16 days, this culture aged into a brown color, and the media had a brown yellow tint to it.

Asymmetrical orange (figure 1C) grew similar to the simple orange for the first 3 days. After 3 days, distinct sectoring appeared. The sectors grew at different rates and were a darker orange. This caused the culture to be asymmetrical. The mycelium was completely vegetative. After 14-16 days, the dark orange sectors of the culture aged into a brown color, and the media had a brown yellow tint to it.

Concentric circle white (figure 1D) grew very fast. It had varying numbers of sectors. For the first 2 days, it grew similar to simple orange. From days 2-7, the culture had white and orange concentric circles and sectors would appear. After 7 days, the culture would reach the edge of the petri plate. The culture itself stayed fairly symmetrical. The mycelium on this type culture appeared fuzzy. The orange circles would turn brown after 14 days, and the media turned into a brown yellow color.

Asymmetrical white (figure 1E) had properties of concentric circle white (figure 1D) and patchy orange (figure 1B). The culture grew exactly like concentric circle white except the sectors took the characteristics of patchy orange (1B). The sectors were severely stunted and the growth never reached the edge of the petri dish. The orange areas of the culture turned brown after 14-16 days, and the media became tinted a yellow brown color during this time period.

A few cultures took on a random growth pattern (figure 1F). They sectored into many different arrangements, and the growth pattern was unpredictable. After 14-16 days, these cultures also tinted the media a yellow brown color.

EP 155 looked very similar to figure 1D, concentric circle white, in culture on PDA without the sector. EP 155 grew very fast and stayed fairly symmetrical and didn't discolor the media after time had passed. EP 4 didn't look like any of the Tatum samples in culture. EP 4 grew slowly with a wrinkled brown-orange texture only sprouting aerial hyphae in the center of the culture on PDA characteristic of the JR phenotype. EP 4 slightly discolored the media to have a yellow tint to it after 2 weeks had passed. The culture itself also got darker as it aged. Figure 2 shows examples of EP 155 and EP 4 cultures that were 14 days old.

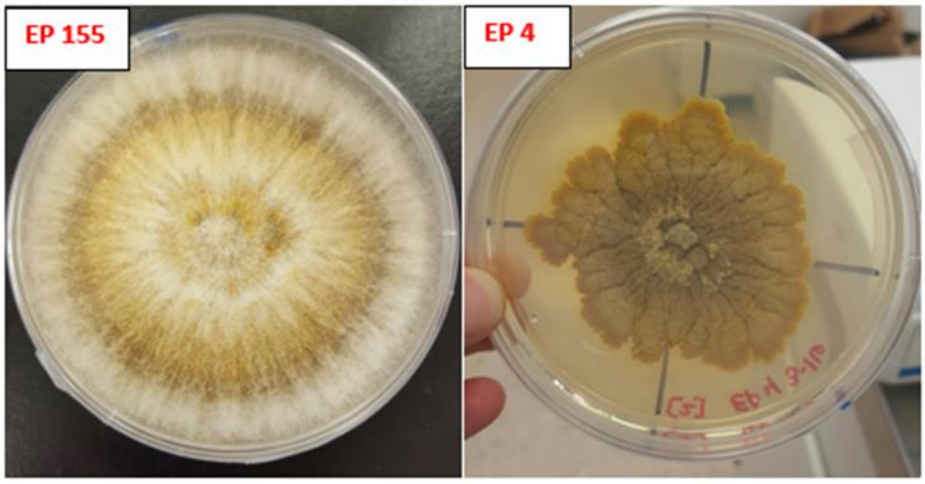

Figure 2. EP 155 and EP 4 cultures at 14 days old. EP155 is the left hand picture in the figure, and EP 4 is the right hand culture in the figure.

Growth analysis

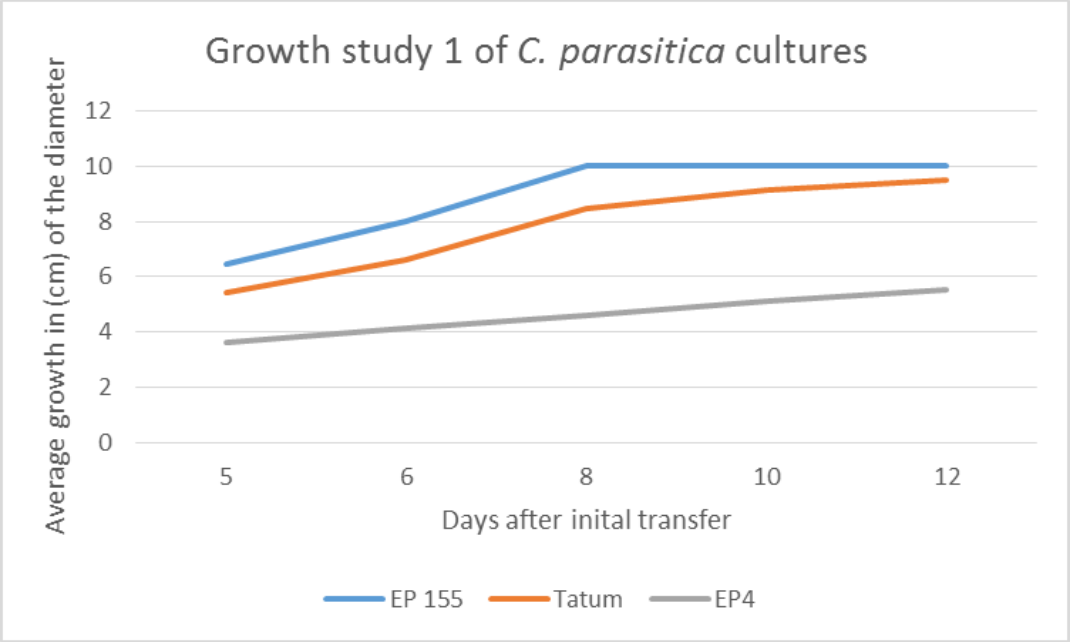

Figure 3. Growth study 1 line graph. Average growth of the EP 155, Tatum, and EP 4 samples of the first growth rate trial from day 5 to day 12.

Figure 3 illustrates that the Tatum sample in trial 1 grew at a rate in between those of EP 4 and EP 155. The samples were grown in culture on petri dishes that were 10 cm in diameter. By day 8, all of the EP 155 cultures grew to cover the complete petri dish. Therefore growth rates were stalled at 10 cm for the remainder of the study for EP 155. The Tatum culture continued to grow but none of the 9 cultures reach a 10 cm diameter both vertically and horizontally. EP 4 continued to steadily grow but never reached an average diameter of 6 cm.

One-way ANOVA analysis was performed on the data for the means of the culture diameters for day 5. The results are summarized in table 1. The null hypothesis for the one-way ANOVA is that there is no difference between the mean of the diameters of EP 155, EP 4, and the Tatum cultures.

Table 1.One-way ANOVA and Tukey HSD results for the means of EP 155, EP 4, and the Tatum sample for day 5.

ANOVA Summary day 5 Trial 1			
Sum of Squares	49.03	F-ratio	53.73
Degrees Freedom	27	P-Value	<0.0001
Conclusion	Reject null hypothesis		

Tukey HSD day 5 trial 1	
EP4 vs EP 155	P-value<0.01
EP4 vs Tatum	P-Value<0.01
EP 155 vs Tatum	P-value<0.01

The ANOVA results indicated to reject the null hypothesis. This implied that the means of the diameters for the EP 155, EP 4, and the Tatum cultures were not equal with a P-value of less than 0.0001. The Tukey HSD test was then performed to find where the individual differences were among each of the mean diameters of EP 155, EP 4, and the Tatum cultures. The results found that the mean diameters of all three cultures were statistically different from one another using an alpha of 0.01 (table 1).

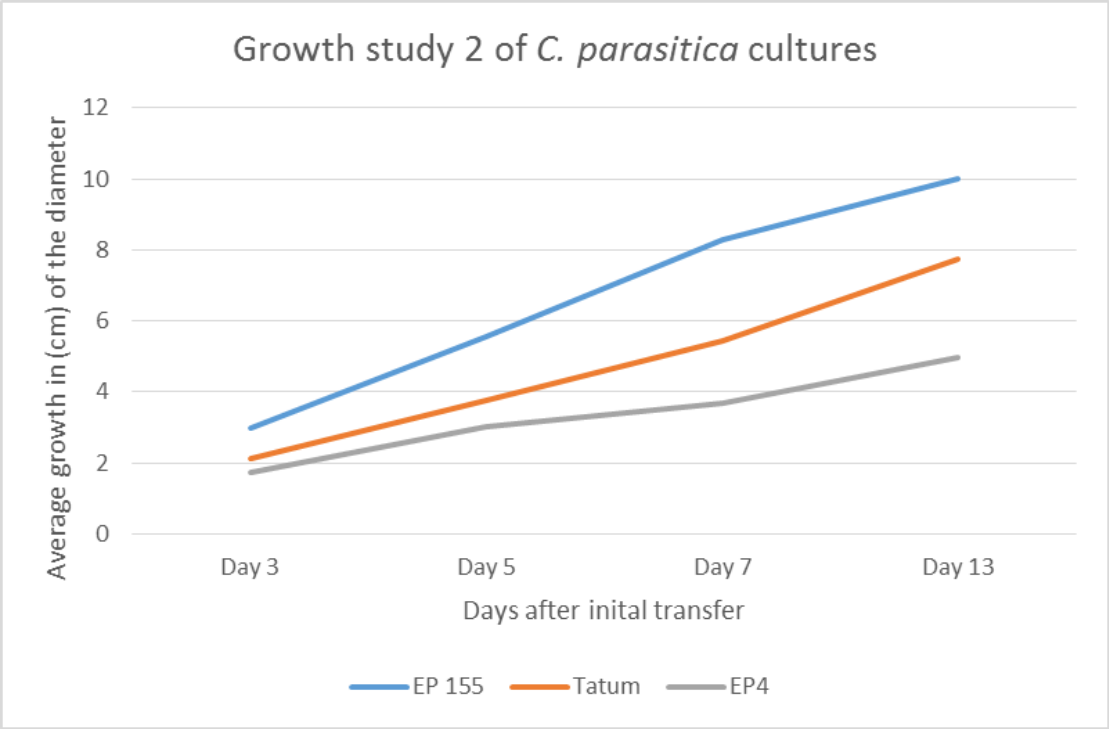

Figure 4. Growth study 2 line graph. Average growth of the EP 155, Tatum, and EP 4 samples of the second growth rate trial from day 3 to day 13.

Figure 4 illustrates that in the second growth rate trial, the Tatum cultures grew at a rate in between EP 155 and EP 4. This was consistent with the results from the first growth rate trial illustrated by figure 3. The mean of the diameters for the Tatum cultures in the second growth study is notably lower than the first study. This can be attributed to random sampling with the many variations of morphology and large amounts of sectoring that can be seen in figure 1.

One-way ANOVA analysis was performed on the data for the means of the culture diameters for day 5. The null hypothesis for the one-way ANOVA is that there is no difference between the mean of the diameters of EP 155, EP 4, and the Tatum cultures. The results are summarized in table 2.

Table 2. One-way ANOVA and Tukey HSD results for the means of EP 155, EP 4, and the Tatum sample for day 5.

ANOVA Summary day 5 trial 2			
Sum of Squares	44.84	F-ratio	41.32
Degrees Freedom	29	P-Value	<0.0001
Conclusion	Reject null hypothesis		

Tukey HSD day 5 trial 2	
EP4 vs EP 155	P-value<0.01
EP4 vs Tatum	P-value<0.05
EP 155 vs Tatum	P-value<0.01

The ANOVA results indicated to reject the null hypothesis. The Tukey HSD tests results for day 5 on trial 2 were that the means of the diameter for all three samples were different from one another (table 2). EP 4 vs EP 155 had a P-value < 0.01. EP 4 vs Tatum had a P-value < 0.05. EP 155 vs Tatum had a P-value < 0.01.

Apple virulence assay

Figure 5. Day 5 apple virulence assay. Photograph of apples taken on day 5 showing the necrotic lesions around inoculation site.

The apples in figure 5 illustrate the relative size of the necrotic lesions around the inoculation site. On day 5 EP 155, the top row of apples, has significant necrosis on all three of the apples. The Tatum sample on the middle row had a small amount of necrosis around the inoculation site. EP 4 didn't have any notable necrosis. These samples appeared just like the control apples that were inoculated with just a PDA agar plug. The diameter of the necrotic spot was used for statistical analysis since the apples were of uniform size. Statistical analysis was done using a

one-way ANOVA using the mean of the horizontal and vertical diameter of the necrotic lesions including the hole made by the cork borer. The results are summarized in table 3.

Table 3. Apple One-way ANOVA and Tukey HSD summary.

ANOVA Summary apples day 5			
Sum of Squares	1.205	F-ratio	11.34
Degrees Freedom	8	P-Value	0.0092
Conclusion	Reject null hypothesis		

Tukey HSD apples day 5	
EP4 vs EP 155	P-value<0.01
EP4 vs Tatum	Non-significant
EP 155 vs Tatum	P-value<0.05

The results for the one-way ANOVA for day 5 of the apple virulence assay was to reject the null hypothesis. The P-value was 0.0092. The means of the diameter of the necrotic lesions for the three treatment groups were not equal. The Tukey HSD test indicated that there was a significant difference between EP 4 and EP 155 with a P-value<0.01. There was also a significant difference between EP 155 and Tatum with a P-value<0.05. EP 4 and Tatum were found to not be significantly different.

Figure 6. Variation of apples on day 10. Side by side comparison of the necrotic lesions of EP 155, Tatum, EP 4, and a control apple at day 10.

Figure 6 illustrates the variation of necrotic lesions on the apples caused by the inoculation of the EP 155, Tatum, and EP 4 strains of *C. parasitica*. Over all the EP 155 apples showed significant necrotic lesions that were all symmetrical and dark. The Tatum apples all grew asymmetrically with necrotic lesions were lighter than EP 155 or EP 4. At day 10, all of the EP 4 apples had small dark symmetrical necrotic spots. The 3 apples used for the control did not show any signs of enlarging necrotic lesions throughout the experiment. The one-way ANOVA and Tukey HSD test are summarized in table 4.

Table 4. Apple one-way ANOVA and Tukey HSD summary.

ANOVA Summary apples day 10			
Sum of Squares	5.54	F-ratio	34.32
Degrees Freedom	8	P-Value	0.00052
Conclusion	Reject null hypothesis		

Tukey HSD apples day 10	
EP4 vs EP 155	P-value<0.01
EP4 vs Tatum	P-value<0.05
EP 155 vs Tatum	P-value<0.05

The results for the one-way ANOVA for day 10 of the apple virulence assay was to reject the null hypothesis. The P-value was 0.00052. The means of the diameter of the necrotic lesions for the three treatment groups were not equal. The Tukey HSD test indicated that there was a significant difference between EP 4 and EP 155 with a P-value<0.01. There was also a

significant difference between EP155 and Tatum with a P-value<0.05. EP 4 and Tatum were found be significantly different with a P-value<0.05.

Oak branch inoculation

The water oak inoculation produced no significant results. The control-inoculated excised branches were compromised. The top layer of bark was removed to look for characteristic discoloration attributed to canker formation. The control, Tatum, and EP 4 branches had been completely discolored. The wood was tinted brown instead of the characteristic cream color expected. EP 155 did provide some branches with the expected discoloration pattern (figure 7) as well as branches that were completely tinted brown. The slightly red tinted oval surrounding the wound from inoculation was the expected zone of discoloration that could be measured and compared to estimate canker formation ability in a native oak species of the three cultures.

Figure 7. EP 155 excised branch. The expected discoloration pattern on the EP 155 excised branch 20 days after inoculation.

Reasons for the complete discoloration of the control, Tatum, and EP 4 branches are unknown. However, thickness of the branches used and duration of time before measuring the discoloration under the bark are possible reasons why the branches were compromised.

For the first 19 days, the branches were observed for evidence of sporulation and any signs or symptoms of being infected with *C. parasitica*. Throughout the 19 day period, no evidence of sporulation was found on any of the branches. The branches were excised during their dormant period and were kept in a dark environment. For these reasons, no sporulation was expected to form. No symptoms were observed from the outer layer of any of the branches, but signs of mycelial growth were observed in and around the wound of inoculation from the branches that were inoculated with a culture.

CHAPTER IV

CONCLUSION

Culture analysis

The Tatum cultures grew with many different morphologies with differences ranging from pigment, sporulation, sectoring, and growth rate. The growth rate study concluded that using an alpha of 0.05, all the cultures were significantly different from one another at day 5. The conclusion for the growth was that EP 155, EP 4, and the Tatum cultures are likely all 3 different strains of cultures.

The range of the morphology, the large amount of sectoring, and variance within the isolates from the Tatum tree can be contributed to many causes such as genetic mutation or a dsRNA. However, it is known that hypovirulent *C. parasitica* field isolates demonstrate an extensive range of variability in virulence levels and in the strength and combination of hypovirulent characteristics (Chung 1994). Not only does the morphology of the culture depend on the dsRNA that is present but also the genetic components of the virulent strain of *C. parasitica*. Therefore, field isolates with a range of genetic variability in both of these components have the tendency to express a range of morphologies. This has been demonstrated well by Dr. Boashan Chen and Dr. Donald Nuss at the University of Maryland in 1999 by recording the morphological changes in two virulent strains of *C. parasitica,* while using infectious cDNA clones of known Cryphonectria hypoviruses (Chen 1999). They found that different virulent isolates reacted differently to the same cDNA clones. Sometimes the cDNA clone would minimally affect one

virulent strain's morphology but severely affect another. For this reason, morphology can be useful to find isolates to test for the presence of a dsRNA but shouldn't be the only method used to look for hypovirulence.

Apple virulence assay

The virulence assay works by giving a visual measurement of the spread of oxalate production at the edge of the cultures. The lack or absence of oxalate production has been a key factor in whether or not a strain is deemed as hypovirulent or virulent (Havir 1983). The virulence assay on the apples has been commonly used as a surrogate to test for virulence of all the isolates on American chestnut bark, because this process has consistently produced the same results as inoculating branches of the American chestnut. The apple virulence assay is less time consuming. The apples are also more practical for large scale studies and studies without access to American chestnut branches (Fullbright 1984).

The results of the ANOVA and Tukey HSD using an alpha of 0.05 for day 5 stated that there wasn't a difference between EP 4 and the Tatum sample, but the Tatum and EP 155 were different. However, on day 10 the results were that all three cultures were significantly different. These results are conclusive that EP155 and the Tatum samples are not the same. The Tatum sample doesn't produce a necrotic spot on the apples as large as EP 155.Therefore, the Tatum sample would not produce as virulent of canker as EP 155 on the American chestnut. However, EP 4 produced very little necrotic lesions on the apples compared to both other isolates at day 10. The Tatum samples grew asymmetrical on the petri plates as well as in the flesh of the apples. Both EP 155 and EP 4 grew symmetrical on the media and in the flesh of the apples. This may

indicate that the sectors that appeared in culture have the potential to have different levels of oxalate production correlated to them.

Discussion and future project goals

The Tatum sample overall seems to be a consistent intermediate of the very hypovirulent EP 4 and the American standard virulent strain EP 155. Further studies need to be completed to get conclusive results. Using PCR and known primers for several known Cryphonectria hypoviruses, it is possible to prove that the Texas strain is in fact hypovirulent (Hiremath 1986). The breakdown of the sequences can be compared to hypovirulent sequences that have been found in Louisiana and Arkansas (Wallace 1970). There are several other tests that have been described to estimate spore production which is an important characteristic especially if biological control through hypovirulence is a goal (Lee 1992), (MacDonald 1991). The oak branch inoculation should be repeated with several more hypovirulent strains of *C.parasitica*, and with branches of several local oak to get an idea how this pathogen interacts with the oak species in the ecology of Texas forests.

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